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Abstract: Cancer stem cells may mediate therapy resistance and recurrence in various types of cancer, including glioblastoma. Cancer stemlike cells can be isolated from long-term cancer cell lines, including glioma lines. Using sphere formation as a model for cancer cell stemness in vitro, we derived sphere cultures from SMA-497, SMA-540, SMA-560, and GL-261 glioma cells. Gene expression and proteomics profiling demonstrated that sphere cultures uniformly showed an elevated expression of stemness-associated genes, notably including CD44. Differences in neural lineage marker expression between nonsphere and sphere cultures were heterogeneous except for a uniform reduction of α -III-tubulin in sphere cultures. All sphere cultures showed slower growth. Self-renewal capacity was influenced by medium conditions but not nonsphere versus sphere culture phenotype. Sphere cultures were more resistant to irradiation, whereas both nonsphere and sphere cultures were highly resistant to temozolomide. Nonsphere cells formed more aggressive tumors in syngeneic mice than sphere cells in all models except SMA-560. There were no major differences in vascularization or infiltration by T cells or microglia/macrophages between nonsphere and sphere cell-derived tumors implanted in syngeneic hosts. Together, these data indicate that mouse glioma cell lines may be induced in vitro to form spheres that acquire features of stemness, but they do not exhibit a uniform biologic phenotype, thereby challenging the view that they represent a superior model system.

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How stem-like are sphere cultures from long-term cancer cell lines: lessons from mouse glioma models

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Running title: Do mouse glioma cell lines contain stem cells?

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Mushfika Ahmad and Karl Frei performed the cell culture and animal experiments. Kerstin Kaulich and Guido Reifenberger did the gene expression analyses. Anja Stefanski and Kai Stühler performed the proteomic studies. Edith Willscher and Hans Binder did all bioinformatic and biostatistical analyses. Michael Weller and Patrick Roth designed and supervised the study.

Abstract

Cancer stem cells may mediate therapy resistance and recurrence in various types of cancer, including glioblastoma. Cancer stem-like cells can be isolated from long-term cancer cell lines, including glioma.

Using sphere formation as a model for cancer cell stemness in vitro, we derived sphere cultures from SMA-497, SMA-540, SMA-560 and GL-261 glioma cells. Gene expression and proteomics profiling demonstrated that sphere cultures uniformly showed elevated expression of stemness-associated genes, notably including CD44. Differences in neural lineage marker expression between non-sphere and sphere cultures were heterogeneous except for a uniform reduction of β -III-tubulin in sphere cultures. All sphere cultures showed slower growth. Self-renewal capacity was influenced by medium conditions, but not non-sphere versus sphere culture phenotype. Sphere cultures were more resistant to irradiation whereas both non-sphere and sphere cultures were highly resistant to temozolomide. Non-sphere cells formed more aggressive tumors in syngeneic mice than sphere cells in all models except SMA-560. There were no major differences of vascularization or infiltration by T cells or microglia/macrophages between non-sphere and sphere cell-derived tumors.

Mouse glioma cell lines may be induced in vitro to form spheres that acquire features of stemness, but do not exhibit a uniform biological phenotype, challenging the view that they represent a superior model system.

List of abbreviations: CNPase, 2',3'-cyclic-nucleotide 3'-phosphodiesterase; DAB, 3,3'-diaminobenzidine; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco`s modified Eagle medium; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; FACS, fluorescence-activated cell sorting; FC, fold change; FCS, fetal calf serum; FGF, fibroblast growth factor; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; Gy, Gray; H&E, hematoxylin and eosin; NB, Neurobasal A medium; NS, non-sphere cultures; P, probability; PBS, phosphate-buffered saline; PE, phycoerythrin; PFA, paraformaldehyde; rpm, rounds per minute; SC, sphere (stem-like) cultures; SEM, standard error of the mean; SFI, specific fluorescence index; SMA, spontaneous mouse astrocytoma; TMZ, temozolomide; β IIIIT, beta three tubulin; kDa, kilodalton

Introduction

Glioblastomas are highly aggressive intrinsic brain tumors that may develop at all age groups, with incidence steadily increasing with age. Despite multimodal treatment of surgery followed by radiotherapy and alkylating agent chemotherapy, the median survival of glioblastoma patients is still in the range of less than one year in population-based studies (1). Effective treatments may be impeded by the high morphological, phenotypic and genetic cellular diversity.

A specific subpopulation of tumor cells, variably termed glioma stem cells or glioma-initiating cells, has been identified in human glioblastomas and has been proposed to confer therapy resistance and tumor progression despite multimodal therapy (2-4).

Glioma stem cells are believed to be responsible for tumor initiation and growth due to their unlimited self-renewal capacity and the ability to give rise to more differentiated tumor cells in the tumor bulk. Furthermore, they have been proposed to express specific DNA damage response proteins as well as drug efflux proteins in response to radiotherapy and chemotherapy with DNA alkylating agents such as temozolomide (TMZ) (5-7).

Neural stem cell culture conditions have been used as a standard method to isolate and selectively expand glioma stem cells as tumor spheres that resemble neurospheres in culture, not only from resected primary gliomas *ex vivo*, but also from C6 rat and U373MG, A172 and U87MG human glioma cell lines (2-4, 8, 9). Reports on the size of the cell population isolated from the C6 rat glioma cell line that displayed these stem cell characteristics varied from 4% to 87% (9, 10). Similar to neural stem cells, the putative

cancer stem cells from glioma cell lines have been shown to be capable of clonal growth, to exhibit multi-lineage differentiation potential, and to share the expression of various neural stem/progenitor cell markers, including CD133, nestin, Musashi-1 and Sox2 (8, 10-13). These neural stem cell characteristics as well as their *in vivo* tumorigenicity are among the criteria used to identify glioma stem cells.

The best choice of experimental model for glioma research depends on the type of scientific question to be addressed (14). GL-261 and SMA cell lines are the most commonly used syngeneic mouse glioma models (15-17). Here, we systematically addressed the question whether sphere cultures can be derived from these cell lines and whether these preferentially exhibit features of stemness, including enhanced self-renewal, stem cell marker expression, multi-lineage differentiation potential, resistance to radiotherapy and chemotherapy, and tumorigenicity *in vivo*.

Materials and Methods

Materials

TMZ (parental grade 2577461) was kindly provided by MSD Switzerland (Zug, Switzerland). Primary antibodies for flow cytometry included: anti-human/mouse CD44 (working dilution of 1:100) (eBiosciences, San Diego, CA), mouse anti-prominin (CD133)-1-phycoerythrin (PE) (1:100) (Miltenyi Biotec, Bergisch Gladbach, Germany), mouse IgG2bk isotype control (eBiosciences), phycoerythrin (PE)-conjugated mouse IgG isotype control (BD Biosciences, San Jose, CA), and fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (BD Bioscience). The concentrations of isotype controls were adjusted to have the same IgG concentration as the specific antibodies. For immunofluorescence staining, the following primary antibodies were used: monoclonal mouse anti-nestin IgG1 (1:200) (AbD serotec, Düsseldorf, Germany), polyclonal rabbit anti-Musashi-1 (1:400) (Chemicon, Zug, Switzerland), polyclonal rabbit anti- β -III-tubulin (β IIIT) IgG (1:1000) (Abcam, Cambridge, UK), monoclonal mouse anti-2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase) IgG1 (1:100) (Chemicon), polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) IgG (1:1000) (Dako, Baar, Switzerland), and monoclonal rabbit anti-Ki-67 IgG (1:100) (Epitomics, Nunningen, Switzerland). Alexa Fluor®-488-conjugated goat anti-rabbit IgG (Invitrogen, Basel, Switzerland) or anti-mouse IgG (both 1:100) (Invitrogen) were used as secondary antibodies. For immunohistochemistry of mouse gliomas *in vivo*, the following primary antibodies were used: anti-human/mouse CD44 (1:1000) (eBiosciences), polyclonal rabbit anti- β IIIT IgG (1:1000) (Abcam), rat anti-CD3 (1:1000) (BD Bioscience), anti-

CD11b (1:1000) (BD Bioscience), anti-CD31 (1:50) (BD Bioscience) and anti-Ki-67 (1:100) (Epitomics).

Cell culture

Murine SMA-497, SMA-540 and SMA-560 cells were kindly provided by Dr. D.D. Bigner (Durham, NC). GL-261 cells were obtained from the American Type Culture Collection (Rockville, MD). These cell lines are commonly cultured as adherent monolayers in Dulbecco's modified Eagle medium (DMEM) (Gibco, Life Technologies, Zug, Switzerland) supplemented with 10% heat-inactivated FCS (Biochrom KG, Zug, Switzerland) and 2 mM glutamine (Biochrom KG) (DM medium), herein referred to as non-sphere cultures. Sphere cultures were generated from non-sphere cultures by placing the cells in Neurobasal (NB)-A medium (Gibco) supplemented with 2% B27 (Gibco), 20 ng/ml basic fibroblast growth factor (FGF) and 20 ng/ml epidermal growth factor (EGF) (Peprotech, London, UK), 6.3 U/ml heparin (Sigma, St. Louis, MO), and 2 mM glutamine (NB medium) at a density of 50,000 cells/ml. Sphere cultures were cultured until the 3rd passage prior to use for experiments, with a passage duration of approximately 7 days.

Cell growth and doubling times

Non-sphere (2,000/ml) or sphere (20,000/ml) cells were seeded in 25 cm² flasks (TPP Techno Plastic Products AG, Trasadingen, Switzerland) in their respective medium. Every day for non-sphere and every two days for sphere cultures, an aliquot of the cells was dissociated into single cell suspensions using TrypLEExpress cell dissociation

reagent (Gibco), stained with 0.4% trypan blue, and viable cells were counted using Neubauer chambers (VWR International AG, Dietikon, Switzerland). Cell counts from three separate experiments were obtained.

Limiting dilution assay

Non-sphere cells were seeded in duplicate in 0.5 ml DM medium in 24 well plates (Faust Laborbedarf AG, Schaffhausen, Switzerland) at the following cell numbers per well: 250, 83, 28, 9, 3 and 1. Sphere cells were also seeded in duplicate in 0.5 ml NB medium at the following cell numbers per well: 1,000, 250, 63, 16, 4 and 1. A colony was defined by > 25 non-sphere cells and the numbers of colonies were counted at 4-14 days. A sphere was defined by > 5 cells and the number of spheres was counted after 7-20 days. A 10x solution of growth factors (EGF and FGF) and heparin was added to the sphere culture plates to a final concentration of 20 ng/ml of growth factors and 6.3 U/ml of heparin, respectively, every week. To assess the plasticity of non-sphere and sphere cells, the colony-forming and sphere-forming capabilities were also assessed in limiting dilution assays after a switch of conditions, that is, by seeding sphere cells in DM medium and by seeding non-sphere cells in NB medium at the cell numbers indicated above for the respective medium. Results were derived from three separate experiments.

Clonogenicity assays

Non-sphere or sphere cells were seeded at 1,000 cells per well with 5 replicates in 96 well plates, treated as indicated, and then further cultured until the untreated non-

sphere cells had formed colonies and the sphere cultures had reached 100-150 μm in sphere size. Growth factors were supplemented weekly to the NB medium as described above. Then, Alamar Blue (Invitrogen) was added to the plates and incubated for 4 h at 37°C after which the plates were analyzed using a cytofluorometer (Infinite 200PRO, Tecan Group Ltd, Männedorf, Switzerland). Assays were repeated three times. Appropriate control experiments had indicated that the Alamar Blue assay is a reliable surrogate marker of clone or sphere number, but more convenient for high volume pharmacological studies than actual clone or sphere counting.

Flow cytometry

Cells were harvested and dissociated into single cells by TrypLEExpress cell dissociation reagent and resuspended in PBS containing 0.5% FCS and 2 mM ethylenediaminetetraacetic acid (EDTA) (FACS buffer). Cells were stained with primary antibodies for 30 min on ice in the dark. After washing with PBS, cells were resuspended in FACS buffer and stained with secondary antibody (when required) on ice for 30 min. After staining, cells were washed with PBS, resuspended in FACS buffer and analyzed using the Beckman Coulter and Summit 4.3 software (Nyon, Switzerland). Staining intensity was expressed as specific fluorescence indexes derived by dividing signal obtained with specific as opposed to isotype control antibody. Percentages of positively stained cells were measured by setting the gate at 1.3% of the fluorescence (represented by the FL 1 log in the histogram) of the isotype control. Similar results were obtained from two separate flow cytometric analyses.

Immunofluorescence microscopy

To analyze the expression of neuronal stem cell, mature neuronal and glial cell markers, sphere cultures were prepared as cytopsin samples whereas non-sphere cultures were grown on sterilized 11 mm glass coverslips (Assistent 1001/12) coverslips at 5,000 cells in a 24 well plate until they were 80-90% confluent. Spheres that had reached a size of 150-200 μm in diameter were harvested by centrifugation at 700 rpm for 5 min and resuspended in 1200 μl of NB medium. 100 μl of resuspended spheres were cytopsin using the Cytospin 4 (Thermo Scientific, Wohlen, Switzerland) at 650 rpm for 4 min. Cytospun spheres were left to dry for 15 min, fixed and stained as outlined below. Cells were washed in PBS and fixed at room temperature for 10 min in either 4% paraformaldehyde (PFA) or methanol pre-cooled to -20°C , according to the antibody recommendations. After washing, cells fixed in PFA were permeabilized in 0.1% Triton X-100 in PBS for 10 min. Cells were then washed and blocked using blocking solution (Candor Biosciences, Wangen, Germany) for 25 min. Primary antibodies were prepared in antibody dilution buffer (DCS Innovative Diagnostik System, Hamburg, Germany). Cells were stained with primary antibodies for 90 min. Following staining, cells were washed in PBS and stained using secondary antibodies prepared in antibody dilution buffer for 30 min. Cells were then stained with 1 $\mu\text{g/ml}$ 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) at a working dilution of 1:1000 in antibody dilution buffer for the last 10 min. Finally the cells were washed in PBS and mounted on microscope slides (Superfrost plus, Thermo Scientific) using fluorescent mounting medium (Dako). Analysis was performed using the SP5 laser scanning confocal microscope (Leica, Wetzlar, Germany) and LAS-AF Lite software. Positively stained cells were identified by

setting the fluorescence signal intensity used for the positive control for each antibody staining under saturation levels. The P19 neuronal progenitor cell line was used as a positive control for nestin and Musashi-1, cryosections of wild type C57BL/6 mouse brain were used as positive control for GFAP, β IIIIT and CNPase.

Irradiation

Cells were seeded in 0.5 ml medium into 24 well plates for limiting dilution or 96 well plates for clonogenic survival assays. On the next day the plates were irradiated at 0, 1, 3 or 9 Gy using a Co source (Gebrüder Sulzer, Thermische Energiesysteme, 60-Co, Winterthur, Switzerland). Cells were allowed to grow and colonies or spheres were counted at 4-14 days after seeding when colonies of at least 25 non-sphere cells had formed or at 7-20 days after seeding when spheres of at least 5 sphere cells had formed, alternatively, Alamar Blue assays (Invitrogen) were performed.

TMZ exposure

Cells were seeded in 0.5 ml of their respective medium and plated into 24 well plates for limiting dilution assays or 96 well plates for clonogenic survival assays. On the next day the cells were treated with 0 (serum-free), 10, 100 or 1000 μ M TMZ diluted in serum-free DM medium or complete NB medium for 24 h. After that the cells were cultured in fresh complete DM or NB medium and allowed to grow. Colonies or spheres were counted, or Alamar Blue assays were performed as described above.

In vivo experiments

The standard operating procedure and the treatment of all animals were according to the Swiss Cantonal Veterinary office regulations under the Animal license permission number 95/2009 and 235/2012. Following anesthesia a burr hole was drilled in the skull 2 mm lateral to the bregma. The needle of a Hamilton syringe (Hamilton, Darmstadt, Germany) was introduced to a depth of 3 mm. A volume of 2 μ l of PBS single cell suspension was slowly injected into the right striatum. 1,000 non-sphere or sphere cells of SMA-497, SMA-540 or SMA-560 cell lines were implanted into 6 groups of 11 mice per group of VM/Dk mice. Similarly, GL-261 cells were implanted in 2 groups (non-sphere and sphere cells) of 10 mice per group in C57Bl/6 mice. Two mice per group were euthanized using a pre-randomization scheme when any mouse in the experiment became symptomatic in order to perform histological studies to assess tumor growth at an early stage (Table 1). The other 9 mice (8 mice for the GL-261 model) were euthanized when displaying neurological symptoms and were thus used for survival analysis (pooled from two separate experiments) and these along with the rest of the brains collected at termination of the experiment, day 80 after implantation, were used for further immunohistochemical stainings. All brains collected upon euthanization were embedded in cryomoulds in Shandon Cytochrome yellow (Thermo Scientific) and frozen in liquid nitrogen. Tumor incidences were determined using hematoxylin and eosin stainings of 8 mm thick cryosections cut using the Microm HM560 (Microchom HM560, Thermo Scientific) before constructing and analyzing Kaplan Meier survival curves using the log-rank test in GraphPad Prism 5.0 software.

Immunohistochemical analysis

Cryosections were fixed in ice-cold acetone for 10 min, washed in PBS and treated with 3% hydrogen peroxide for 10 min. The sections were then washed and blocked using blocking solution (Candor Biosciences). After blocking the primary antibodies were applied overnight at 4°C. Next, the sections were washed and probed using biotinylated rabbit-anti-rat secondary antibody (Vector) for 30 min at room temperature. The antigen antibody conjugates were then detected by staining with 3,3'-diaminobenzidine (DAB) (Dako) for 1-3 min. The nuclei were stained using hematoxylin for 4 min, washed in water and dehydrated twice in 96% ethanol, then twice in 100% ethanol and three times in xylol before mounting onto coverslips using Eukitt mounting medium. Quantification of proliferating cells (Ki-67), blood vessel density (CD31), T cells (CD3) and microglial cells/macrophages (CD11b) was obtained from 4-6 different areas (depending on tumor size) from 3 tumors each at the terminal stage in an area of 0.35 mm². β IIIT and CD44 levels were not quantified. Statistical significance was calculated using the paired student t-test.

Analysis of gene expression and proteomics data

The generation of transcriptomic and proteomic data is reported in detail in the Supplementary Note. Raw microarray data were calibrated and summarized using the RMA method including quantile-normalization (18, 19). The expression value of each gene was transformed into log₁₀-scale and centered with respect to its mean value averaged over all samples investigated (20). A relative log expression value of zero indicates that the gene is expressed according to its mean expression value, while

positive and negative values refer to over- and under-expression in the data set, respectively.

Gene expression was analysed using the portraying method based on self-organizing map (SOM) data compression. This approach transforms the gene expression patterns of more than 20,000 genes per sample into intuitive quadratic mosaic portraits representing 50x50 grids, in which each tile refers to one microcluster of concertedly expressed genes called 'metagene' (20, 21). Further analyses were performed either in metagene space, e.g., by clustering concertedly expressed metagenes into so-called spot modules, by calculating group-averaged mean portraits for each cell line and sample condition, or by selecting differential features by calculating difference portraits (Suppl. Fig. 1,2) or based on single gene expression data, e.g., by applying gene set enrichment analysis for functional interpretation. The latter approach makes use of the gene set enrichment score (GSZ) which estimates the significance of differential expression of the members of a set of genes compared with the mean expression of all genes in a sample (22). Gene sets were taken from standard gene ontology (GO) categories or from previous publications as indicated below. All analyses were performed using the R-program opoSOM providing basic SOM portraying in combination with a series of data mining methods and comprehensive visualization options (20) (22).

Protein data were processed using the same analysis pipeline as described above for gene expression data except for RMA calibration, i.e., the data were log-10 transformed, centralized and subsequently analyzed using the SOM-portraying method. Suitability of this method for analyzing proteomics data was recently demonstrated (23).

Statistical analysis

For the functional assays, data are usually representative of experiments performed three times with similar results. Statistical significance for the clonogenicity studies after irradiation or TMZ treatments was assessed with a two-way ANOVA test using the GraphPad Prism 5.0 software. Statistical analysis of cell doubling time and immunofluorescence was calculated using two-tailed student t-test with equal variance while immunohistochemistry data were analysed using two-tailed student t-test with unequal variance, due to the heterogeneous nature of tumors. Survival statistics in the *in vivo* studies were calculated using the log-rank test from the GraphPad Prism 5.0 software.

Results

Gene expression and proteomics profiling of sphere versus non-sphere cultures of murine glioma cells in vitro

Sphere cultures were established from non-sphere cultures of all four mouse glioma cell lines by switching the non-sphere cultures to NB medium, which resulted in sphere formation in all cell lines (Fig. 1A). These spheres could be maintained for months under these culture conditions. Microarray-based mRNA expression profiling and mass spectrometry-based proteome analyses were done to assess the gene activity in terms of mRNA and protein abundance, respectively (Supplementary Note). The primary analysis was performed by comparing the expression portraits of all sample classes, i.e., cell lines and culture conditions, obtained from the transcriptome and proteome data, respectively. In contrast to the light microscopic features which were dominated by the culture conditions, the molecular expression landscapes were mainly governed by the respective cell line (Fig. 1B). For example, each of the SMA lines showed similar gene expression largely independent of the non-sphere or sphere culture condition, reflected also in the similarity tree based on mRNA expression profiles, which also outlines that SMA-560 is less related to the other two, more related SMA lines (Suppl. Fig. 3). The proteomic portraits supported this observation for GL-261 whereas the proteomics portraits of the SMA samples revealed clear differences between the sphere culture and non-sphere culture conditions (Figure 1D, E, Suppl. Fig. 4). Non-sphere cultures of the SMA lines were similar whereas GL-261 exhibited a different proteomics profile. Thus, altogether, the similarities between paired non-sphere and sphere cultures

were higher than among non-sphere versus sphere cultures, irrespective of whether GL-261 was included or excluded from the analyses.

Expression of stemness markers

Next we performed differential analyses between the samples cultured under non-sphere versus sphere conditions (Suppl. Fig. 1,2). Both transcriptomics and proteomics data revealed a cluster of mRNA and proteins concertedly down-regulated in non-sphere and upregulated in sphere cultures, depicted as spot profiles in Fig. 1C,E). Selected sets of human embryonic stem cell marker genes (24) were selectively up-regulated in sphere cultures at mRNA level and even more clearly at protein level (Fig. 2). No such changes were observed for other marker sets (data not shown). Changes in stem cell marker expression induced by sphere formation were often not uniform: Notch1 mRNA was up-regulated in SMA-560 (expression fold change, FC=1.8, p=0.06), Sox2 mRNA in SMA-497(FC=1.8, p=0.05), SMA-540 (FC=2.2, p=0.005) and SMA-560 (FC=3.3, p=0.001), and α 6-integrin mRNA in SMA-497 (FC=3.1, p=0.04). α 6-integrin protein was increased in SMA-497 (FC=1.5, p=0.07), SMA-540 (FC=1.5, p=0.02) and SMA-560 (FC=1.8, p=0.03). CD44 induction as a marker of stemness in sphere cultures was confirmed by flow cytometry (Fig. 3A). Apart from SMA-497 non-sphere and sphere cultures, the mouse glioma cell lines were negative for CD133, and CD133 expression decreased when SMA-497 cells were shifted to sphere culture conditions (Fig. 3B). There was also heterogeneous expression of a third stem cell marker, nestin, among non-sphere and sphere cultures: nestin expression decreased in SMA-540 (P=0.0047), increased by trend in SMA-560 (p=0.16), and remained similar in the other two cell lines

(Fig. 3C,D). Finally, Musashi1 was highly expressed in 3 of the 4 cell lines and essentially unaffected by switching from non-sphere to sphere culture conditions (Fig. 3E,F).

Expression of markers of neuroglial differentiation markers

The non-sphere cultures expressed neuroglial differentiation markers at variable levels by immunofluorescence microscopy. The astrocytic marker GFAP was highly expressed in SMA-497 and SMA-560, to a lesser degree in GL-261, but not in SMA-540 (Fig. 4A,B). The neuronal marker β IIIIT was expressed in the SMA lines, but only at very low level in GL-261 (Fig. 4C,D). In contrast, the oligodendroglial marker CNPase was almost uniformly expressed (Fig. 4E,F). The percentages in Fig. 4B,D,F illustrate that many cells co-expressed different markers of the different neuroglial lineages. We then determined whether induction of the sphere culture phenotype was associated with decreased expression of these markers. GFAP increased under sphere culture conditions in SMA-540 ($P=0.0007$) and by trend in GL-261 ($P=0.18$), whereas CNPase remained highly expressed throughout. Only β IIIIT expression uniformly decreased when the cells were induced to undergo sphere formation at the following significance levels: SMA-497 ($P=0.0009$), SMA-540 ($P=0.034$), SMA-560 ($P=0.13$) and GL-261 ($P=0.093$).

Cell growth and clonogenicity of non-sphere versus sphere cultures

Non-sphere cultures of the SMA cell lines had shorter doubling times than sphere cultures at the indicated significance levels: SMA-497 ($P=0.03$), SMA-540 ($P=0.025$), SMA-560 ($P=0.028$). The difference was not significant in GL-261 cells ($P=0.067$).

(Suppl. Fig. 5A-C). Also, quantification of Ki-67 immunolabeling, a proliferation marker, confirmed higher proliferation of non-sphere cultures for SMA-497 ($P=0.019$), SMA-540 ($P=0.005$) and SMA-560 ($P=0.015$), but not for GL-261 ($P=0.3$) (Suppl. Fig. 5D-E). The GSZ score of the gene set 'cell division', taken from the collection of gene ontology (GO) terms 'biological process', in all systems studied confirmed this result at least by trend at the proteomics level: non-sphere conditions were associated with increased activities of the respective genes (Suppl. Fig. 5F).

Next we assessed the ability for self-renewal in the form of adherent colonies for non-sphere cells or of sphere formation for sphere culture cells, using limiting dilution assays. At similar seeding densities, all non-sphere cultures were superior at self-renewal to sphere cultures in their respective native culture conditions (Fig. 6A-D). To distinguish between impact of "cell type" (non-sphere versus sphere cultures) and impact of cell culture conditions (DM versus NB), we performed medium switch experiments in that the non-sphere cells were seeded and cultured in NB medium and the sphere cells in DM medium in the limiting dilution assays. These experiments demonstrated that the DM medium promoted colony formation of sphere cells whereas the colony forming capacity of non-sphere cells diminished to those of sphere cultures when maintained in NB medium (Fig. 6E-H).

Differential sensitivity of non-sphere versus sphere cultures to irradiation or TMZ

We next explored the differential sensitivity of non-sphere versus sphere cultures to single fractions of irradiation at 1, 3 or 9 Gy (Fig. 7) or to increasing concentrations of TMZ (Fig. 8). Effects on survival and repopulation as well as self-renewal properties

were assessed using clonogenic survival and limiting dilution assays. Proliferation of all cell lines was only affected at 9 Gy. Non-sphere and sphere cultures of SMA-497 and SMA-560 were affected similarly whereas SMA-540 sphere cultures ($P=0.26$) and GL-261 non-sphere cultures ($P=0.003$) were relatively more radiosensitive (Fig. 7A-D). Self-renewal of non-sphere cultures of all cell lines was more affected than that of sphere cultures across the full range of cell numbers seeded (Fig. 7E-H). Similar assays were performed to study the effect of TMZ. All cell lines were highly resistant to TMZ. At the highest TMZ concentration, the SMA-497 and SMA-540 non-sphere cells were more sensitive than the sphere cells, but not to a significant extent. In contrast, no difference between non-sphere and sphere cultures emerged for SMA-560 or GL-261 (Fig. 8A-D). The self-renewal capacities of non-sphere and sphere cultures after exposure to TMZ at 100 μ M were unaffected in all four cell lines at all seeding densities (Fig. 8E-H).

Tumorigenicity in syngeneic mice

In order to compare tumorigenicity *in vivo*, 1,000 non-sphere or sphere cells were injected into the right striatum of syngeneic VM/Dk mice for the SMA cell lines or of C57Bl/6 mice for GL-261 cells. In 3 of 4 cell lines, non-sphere cell implantation produced higher tumor incidences and shorter survival times than sphere cell implantation: the significance levels for the differences of the estimated median survivals were: SMA-497 ($P<0.0001$), SMA-540 ($P=0.0026$) and GL-261 ($P=0.0005$) (Fig. 5A,B,D). Only in the SMA-560 cell line did the sphere cell-derived tumors kill the animals earlier than non-sphere cell-derived tumors ($P<0.0001$) (Fig. 5C). Accordingly, when early stages of tumor development were assessed around day 14 in the SMA and

at day 29 in the GL-261 models, non-sphere cells of SMA-497 and SMA-540 and sphere cells of SMA-560 had already developed large tumors and mice were severely symptomatic. Tumors were rather large in GL-261 in both groups at day 29 when the mice became symptomatic (Table 1). Further tumor progression did not necessarily result in tumors with increased diameters in all groups, but instead was associated with multifocal tumor formation that crossed the midline to invade into the contralateral hemisphere. Hematoxylin and eosin stainings of all tumors at the terminal stage revealed a densely packed mass of pleomorphic cells. Apart from SMA-497 sphere cells which did not form a solid primary tumor, but grew diffusely from the beginning, the other non-sphere and sphere cells formed a solid lesion from which streams of tumor cells and single cell infiltration into normal tissue evolved, resulting in secondary tumors and proliferation around perivascular spaces distant from the primary lesion (Suppl. Fig. 6). The largest tumor diameters and highest incidence of multifocal lesions were seen in the SMA-540 non-sphere and GL-261 sphere cell tumors. In end-stage tumors, proliferation assessed by Ki-67 labeling was similar in sphere and non-sphere cell-derived tumors in SMA-497, but increased in sphere cell-derived tumors of SMA-560 and GL-261. Ki-67 labeling was localized mainly at the periphery of SMA-497 non-sphere cell tumors whereas both non-sphere and sphere cell tumors in the other cell lines had a uniform distribution of proliferating cells within the tumor.

CD44 and β IIIT levels were assessed to explore whether some of the differences between non-sphere and sphere cultures *in vitro* were maintained when these cells were passaged *in vivo*. This was not the case since CD44 was uniformly expressed in all non-sphere and sphere culture tumors, but not in adjacent brain. In contrast, β IIIT

was barely detectable in the tumors, but strongly expressed in normal brain, corresponding to the sphere culture rather than the adherent phenotype *in vitro*. CD31 labeling reflecting vessel density was similar in both non-sphere and sphere cell tumors of all cell lines, with significantly higher vessel density seen in the sphere cell group only in SMA-560. The SMA-560 tumors were also the most vascularized of all models in general. CD3-labeled T cells were found localized at the tumor periphery (Suppl. Fig. 6A) and in perivascular regions (Suppl. Fig. 6C). They were present at higher levels in non-sphere tumors of the SMA models and in the sphere tumors of GL-261. CD11b infiltration was more prominent in non-sphere than in sphere cell tumors in the SMA models, but not in GL-261 where sphere cell tumors showed higher infiltration. It was found within the tumors and outside the tumor (Suppl. Fig. 6C) and sometimes in higher densities in areas surrounding blood vessels in GL-261 tumors (Suppl. Fig. 6D). Overall there was no association of the extent of immune cell infiltration with outcome.

Discussion

The notion that cancers, although of monoclonal origin, are heterogeneous and not composed of a monomorphic population of cells is now widely accepted. Various approaches have been used to characterize and derive a population of cancer stem cells from various types of tumors, including brain tumors, in particular glioblastoma. These cancer stem cells have been considered an often quiescent, slowly proliferating pool of cells exhibiting resistance to conventional modes of non-surgical therapies, that is, irradiation or genotoxic pharmacotherapy. Stem cells have not only been isolated from freshly resected tumors *ex vivo*, but have also been derived from long-term glioma and other cancer cell lines, and have been proposed to serve as superior model systems to study biology of disease in terms of preserving the invasive phenotype and genomic profile of the original tumor (4, 25-27).

Here we addressed the usefulness of stem cells defined by their sphere-forming capacity in a panel of four long-term mouse glioma cell lines. SMA-497, SMA-540 and SMA-560 cells were generated from a spontaneous murine astrocytoma detected in a VM/Dk mouse (15, 17). GL-261 cells were derived from a tumor generated by implantation of methylcholanthrene pellets in C57Bl/6 mouse brains (16, 28). Their neuroglial origin was confirmed here by the differential, often co-expression of neuroglial lineage markers such as GFAP, β IIIIT and CNPase (Fig. 4). We found that even after decades of passaging under differentiating (serum-containing) culture conditions, all mouse glioma cell lines had retained the plasticity to grow in spheres with the option of long-term expansion (Fig. 1A). The sphere culture transcriptomes were uniformly

enriched in some stemness-associated genes, including CD44, and such changes from non-sphere to sphere cultures were mostly confirmed at the proteome level.

Up-regulation of one candidate stem cell marker, CD44, a cell surface adhesion protein thought to play a role in tumor progression, has been associated with increased histopathological grade and poorer prognosis in human gliomas as well as increased cell migration and invasiveness in A172 glioma cells (29, 30). In addition, the expression of CD44 in glioma stem cells has been shown to be controlled by the transforming growth factor- β signalling pathway, which is a target for glioma therapy because of its role in tumor growth (31). Here, CD44 expression increased in 3 of 4 cell lines under sphere culture conditions, but CD44 was almost lost when SMA-540 were induced to assume a sphere culture phenotype (Fig. 3A). The classical glioma stem cell marker CD133, which is increasingly questioned regarding its usefulness to delineate stem cells of human glioblastomas (32-34), did not distinguish between sphere and non-sphere cultures in this panel of mouse glioma cell lines (Fig. 3B). Finally, neither nestin nor Musashi-1 expression correlated with the non-sphere versus sphere culture phenotype (Fig. 3C-F). Importantly, our study does not challenge the value of any of these markers to detect stemness because we do not propose that the sphere cultures generated by changing the culture conditions should be considered stem-like.

GFAP, β IIIIT and CNPase are commonly used as markers of astrocytic, neuronal, and oligodendroglial differentiation, although GFAP expression is also a radial glial neural stem cell marker (35). Our analysis of differentiated neural and glial markers showed that sphere culture conditions did not exclusively enrich for a less differentiated, stem-like phenotype, as previously observed (36), as evidenced by the expression of GFAP

and CNPase. Of note, the anti-CNPase antibody used here recognizes both the 46kDa CNPase isoform, present in mature oligodendrocytes, and the 48kDa, both of which were found to be expressed in oligodendrocyte precursor cells in the normal rat brain as well as in C6 glioma cells (37, 38). By contrast, sphere cultures of all cell lines showed decreased expression of the mature neuronal marker β IIIIT (Fig. 4).

We find that sphere cultures have slowed growth, which we attribute to the deprivation of serum-derived mitogenic stimuli (Suppl. Fig. 5). Unexpectedly, sphere cultures from all four cell lines displayed lower self-renewal capacity than non-sphere cultures in limiting dilution assays. Medium switch experiments disclosed that the type of medium rather than a stable sphere culture versus non-sphere trait conferred the increased self-renewal (Fig. 6).

Relative resistance to radiotherapy and chemotherapy, the principal treatment modalities for human glioblastoma patients (39), is a conceptual hallmark of glioma stem cells. Putatively related to slowed growth kinetics, sphere cultures were uniformly less radiosensitive than non-sphere cultures (Fig. 7), but both sphere and non-sphere mouse glioma cell cultures were resistant to clinically relevant concentrations of TMZ (Fig. 8).

Recent studies have highlighted the limitations of using sphere culture conditions to enrich for cancer stem cells (36, 40). *In vivo* studies indicated that sphere cells with low self-renewal capacity *in vitro* generated rapidly developing tumors with higher penetrance compared to more 'stem-like' cells that possessed higher *in vitro* self-renewal capacity (41). Using a limiting cell implantation number approach in order to critically assess the tumorigenicity of sphere cultures versus non-sphere, we find that

sphere cultures were not enriched in cells with higher tumorigenic potential *in vivo* (Fig. 5). In fact, non-sphere cultures were more tumorigenic in all but the SMA-560 cell line. Moreover, we did not observe stable morphological phenotypes defined by proliferative activity or vascular density that would have allowed distinguishing between non-sphere and sphere cell-derived tumors derived from the same cell line, suggesting that the differential culturing *in vitro* confirmed differential properties for initial seeding and growth *in vivo*, but did not produce genuinely different tumors. Thus, living in a sphere rather than as a monolayer *in vitro* is a state rather than a trait of a mouse glioma cell. Accordingly, the differences in CD44 expression between non-sphere and sphere cultures were not maintained *in vivo*, and, interestingly, the loss of β IIIIT *in vivo* may indicate that sphere cultures are closer to the *in vivo* situation than non-sphere cultures. Furthermore, there also seemed to be no difference in immune responsiveness and host cell infiltration defined by the numbers of CD3-positive T cells or of CD11b-positive cells, possibly representing myeloid suppressor cells.

In conclusion, this study provides an extensive characterization of the most commonly used syngeneic mouse glioma models that should guide researchers in selecting such models for their studies. We find no evidence that studies performed with sphere cultures derived from long-term cell lines are biologically more meaningful than studies performed using standard monolayer cultures, at least in the investigated mouse glioma lines. Future studies may need to define whether this holds true for other glioma cell line model systems, too. Importantly, our data must not be used to argue against the increasing use of human stem-like tumor cell lines immediately derived from freshly resected tumor specimens.

Conflict of interest: MA, KF, EW, AS, KK, HB and KS report no conflict of interest. GR has received a research grant from Roche and honoraria for advisory boards from Roche and Merck Serono. PR has received honoraria for advisory boards from Roche, Molecular Partners and MSD. MW has received research grants from Bayer, Isarna, Merck Serono, MSD and Roche and honoraria for lectures or advisory boards from Isarna, Magforce, Merck Serono, MSD and Roche.

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References

1. Ronning PA, Helseth E, Meling TR, et al. A population-based study on the effect of temozolomide in the treatment of glioblastoma multiforme. *Neuro Oncol* 2012;14;1178-84.
2. Ignatova TN, Kukekov VG, Laywell ED, et al. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *Glia* 2002;39;193-206.
3. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature* 2004;432;396-401.
4. Galli R, Binda E, Orfanelli U, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Research* 2004;64;7011-21.
5. Eramo A, Ricci-Vitiani L, Zeuner A, et al. Chemotherapy resistance of glioblastoma stem cells. *Cell Death Differ* 2006;13;1238-41.
6. Liu GT, Yuan XP, Zeng ZH, et al. Analysis of gene expression and chemoresistance of CD133(+) cancer stem cells in glioblastoma. *Mol Cancer* 2006;5.
7. Bao S, Wu Q, McLendon RE, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 2006;444;756-60.
8. Qiang L, Yang Y, Ma YJ, et al. Isolation and characterization of cancer stem like cells in human glioblastoma cell lines. *Cancer Lett* 2009;279;13-21.

9. Shen G, Shen F, Shi ZJ, et al. Identification of cancer stem-like cells in the C6 glioma cell line and the limitation of current identification methods. *In Vitro Cell Dev-An* 2008;44;280-9.
10. Zhou XD, Wang XY, Qu FJ, et al. Detection of cancer stem cells from the C6 glioma cell line. *J Int Med Res* 2009;37;503-10.
11. Muto J, Imai T, Ogawa D, et al. RNA-binding protein Musashi1 modulates glioma cell growth through the post-transcriptional regulation of Notch and PI3 kinase/Akt signaling pathways. *Plos One* 2012;7.
12. Pellegatta S, Poliani PL, Corno D, et al. Neurospheres enriched in cancer stem-like cells are highly effective in eliciting a dendritic cell-mediated immune response against malignant gliomas. *Cancer Res* 2006;66;10247-52.
13. Wu A, Oh S, Wiesner SM, et al. Persistence of CD133+ cells in human and mouse glioma cell lines: detailed characterization of GL261 glioma cells with cancer stem cell-like properties. *Stem cells and development* 2008;17;173-84.
14. Huszthy PC, Daphu I, Niclou SP, et al. In vivo models of primary brain tumors: pitfalls and perspectives. *Neuro Oncol* 2012;14;979-93.
15. Serano RD, Pegram CN, Bigner DD. Tumorigenic cell culture lines from a spontaneous VM/Dk murine astrocytoma (SMA). *Acta Neuropathol* 1980;51;53-64.
16. Ausman JI, Shapiro WR, Rall DP. Studies on the chemotherapy of experimental brain tumors: development of an experimental model. *Cancer Res* 1970;30;2394-400.
17. Fraser H. Astrocytomas in an inbred mouse strain. *J Pathol* 1971;103;266-70.

18. Binder H, Krohn K, Preibisch S. "Hook"-calibration of GeneChip-microarrays: chip characteristics and expression measures. *Algorithms for molecular biology : AMB* 2008;3;11.
19. Irizarry RA, Hobbs B, Collin F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 2003;4;249-64.
20. Wirth H, Löffler M, von Bergen M, et al. Expression cartography of human tissues using self organizing maps. *BMC Bioinformatics* 2011;12;306.
21. Hopp L, Wirth H, Fasold M, et al. Portraying the expression landscapes of cancer subtypes: A glioblastoma multiforme and prostate cancer case study. *Systems Biomedicine* 2013;1.
22. Wirth H, von Bergen M, Binder H. Mining SOM expression portraits: feature selection and integrating concepts of molecular function. *BioData mining* 2012;5;18.
23. Wirth H, von Bergen M, Murugaiyan J, et al. MALDI-typing of infectious algae of the genus *Prototheca* using SOM portraits. *Journal of microbiological methods* 2012;88;83-97.
24. Wang X. Computational analysis of expression of human embryonic stem cell-associated signatures in tumors. *BMC research notes* 2011;4;471.
25. Li A, Walling J, Kotliarov Y, et al. Genomic changes and gene expression profiles reveal that established glioma cell lines are poorly representative of primary human gliomas. *Molecular cancer research : MCR* 2008;6;21-30.
26. Ernst A, Hofmann S, Ahmadi R, et al. Genomic and expression profiling of glioblastoma stem cell-like spheroid cultures identifies novel tumor-relevant genes associated with survival. *Clin Cancer Res* 2009;15;6541-50.

27. Schulte A, Gunther HS, Phillips HS, et al. A distinct subset of glioma cell lines with stem cell-like properties reflects the transcriptional phenotype of glioblastomas and overexpresses CXCR4 as therapeutic target. *Glia* 2011;59:590-602.
28. Zimmerman HM, Arnold H. Experimental Brain Tumors: II. Tumors produced with benzpyrene. *Am J Pathol* 1943;19:939-55.
29. Yoshida T, Matsuda Y, Naito Z, et al. CD44 in human glioma correlates with histopathological grade and cell migration. *Pathology international* 2012;62;463-70.
30. Wei KC, Huang CY, Chen PY, et al. Evaluation of the prognostic value of CD44 in glioblastoma multiforme. *Anticancer Res* 2010;30;253-9.
31. Anido J, Saez-Borderias A, Gonzalez-Junca A, et al. TGF-beta receptor inhibitors target the CD44(high)/Id1(high) glioma-initiating cell population in human glioblastoma. *Cancer Cell* 2010;18;655-68.
32. Sun Y, Kong W, Falk A, et al. CD133 (Prominin) negative human neural stem cells are clonogenic and tripotent. *PLoS One* 2009;4:e5498.
33. Joo KM, Kim SY, Jin X, et al. Clinical and biological implications of CD133-positive and CD133-negative cells in glioblastomas. *Laboratory investigation; a journal of technical methods and pathology* 2008;88;808-15.
34. Beier D, Rohrl S, Pillai DR, et al. Temozolomide preferentially depletes cancer stem cells in glioblastoma. *Cancer Res* 2008;68;5706-15.
35. Doetsch F, Caille I, Lim DA, et al. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 1999;97;703-16.

36. Pollard SM, Yoshikawa K, Clarke ID, et al. Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. *Cell Stem Cell* 2009;4:568-80.
37. Gravel M, Gao E, Hervouet-Zeiber C, et al. Transcriptional regulation of 2',3'-cyclic nucleotide 3'-phosphodiesterase gene expression by cyclic AMP in C6 cells. *J Neurochem* 2000;75:1940-50.
38. Scherer SS, Braun PE, Grinspan J, et al. Differential regulation of the 2',3'-cyclic nucleotide 3'-phosphodiesterase gene during oligodendrocyte development. *Neuron* 1994;12:1363-75.
39. Weller M, van den Bent M, Hopkins K, et al. EANO guideline for the diagnosis and treatment of anaplastic gliomas and glioblastoma. *Lancet Oncol* 2014;15:e395-e403.
40. Pastrana E, Silva-Vargas V, Doetsch F. Eyes wide open: a critical review of sphere-formation as an assay for stem cells. *Cell Stem Cell* 2011;8:486-98.
41. Barrett LE, Granot Z, Coker C, et al. Self-renewal does not predict tumor growth potential in mouse models of high-grade glioma. *Cancer Cell* 2012;21:11-24.

Figure legends

Figure 1. Gene expression and proteomic profiling of sphere cultures derived

from longterm mouse glioma cell lines *in vitro*. A. Light microscopic features under non-sphere and sphere culture conditions. B,C. Expression profiling: mean SOM gene expression portraits of the cell lines cultured under non-sphere or sphere conditions and of the cell line-specific difference between non-sphere and sphere cultures. The ellipses in the difference portrait of SMA-540 indicate clusters of genes (so-called 'spots') differentially expressed between non-sphere and sphere cultures (B). Their expression levels in the different samples are shown as barplots in C. D,E show the corresponding results for proteomics (NS, non-sphere cultures, SC sphere cultures).

Figure 2. Stem cell marker expression: gene expression and proteomic profiling.

Gene set enrichment analysis using stemness-related genes (24) shows profiles of the enrichment score for three gene sets in all cell systems studied. The sets contain genes differentially upregulated in hESC9 (A, set hESC9), abundant in hESC (B, hESC10) and myc-regulated targets (C, myc2) (NS, non-sphere cultures, SC sphere cultures).

Figure 3. Stem cell marker expression: candidate approach.

A,B. CD44 (A) or CD133 (B) expression were assessed by flow cytometry (CD44/CD133, black lines; isotype control, grey lines, representative of experiments done twice with similar results). SFI and percentages of positive cells are indicated in the right upper corner of each graph. C-F. Nestin (C,D) or Musashi-1 (E,F) expression were determined by

immunofluorescence microscopy and quantified based on DAPI counterstaining from three separate stainings (* $P < 0.01$, paired t-test with equal variance) (size bars in C and E correspond to 50 μm) (NS, non-sphere cultures, SC sphere cultures).

Figure 4. Neuroglial differentiation marker expression. Immunofluorescence microscopy was used to assess GFAP (A,B), β IIIIT (C,D), or CNPase (E,F) expression (non-sphere grey bars, sphere black bars; size bars correspond to 50 μm). Quantitative analysis displays the mean and standard deviation from three experiments (* $P < 0.05$, *** $P < 0.001$, paired t-test with equal variance) (NS, non-sphere cultures, SC sphere cultures).

Figure 5. Tumorigenicity of non-sphere versus sphere cells *in vivo*. A-D. 1,000 non-sphere or sphere cells were implanted into the right striatum of syngeneic mice and the mice were monitored for survival. Results were pooled from two separate experiments (*** $P < 0.001$, ** $P < 0.01$ by log rank test) (NS, non-sphere cultures, SC sphere cultures).

Figure 6. Clonogenicity and spherogenicity of non-sphere versus sphere cells *in vitro*. Colony numbers of non-sphere cultures (open squares, dotted lines) or sphere numbers of sphere cultures (black circles, straight lines) were obtained from 3 limiting dilution assays at 4-14 days after seeding either in their native medium (DM medium for non-sphere and NB medium for sphere cultures (A-D) or in switched medium (non-sphere cells in NB medium and sphere cells in DM medium) (E-H).

Figure 7. Radiosensitivity of non-sphere versus sphere cells *in vitro*. A-D. The effect of 0, 1, 3 and 9 Gy of ionizing irradiation on the proliferation of non-sphere (open squares, dotted lines) versus sphere (filled circles, black lines) cells was assessed by irradiating 1,000 cells plated on 96 well plates and allowing them to recover for 5-14 days in three separate experiments. Assays were stopped when untreated cells became confluent. Quantification of cell density was done by Alamar blue staining. E-H. Limiting dilution assays after irradiation at 0 (open squares, dotted line for non-sphere cells, open circle continuous line for sphere cells) or 9 Gy (closed square, dotted line for non-sphere cells, closed circle, continuous line for sphere cells) were performed. Colonies or spheres were counted 7-20 days after irradiation (n=3, *P<0.05, ***P<0.001, 2 way ANOVA) (NS, non-sphere cultures, SC sphere cultures).

Figure 8. Sensitivity to TMZ of non-sphere versus sphere cells *in vitro*. A-D. The effect of increasing concentrations (0, 10, 100, 1,000 μ M) of TMZ on the proliferation of non-sphere cells (open squares, dotted lines) versus sphere cells (filled circles, black lines) was assessed by treating 1,000 cells plated on 96 well plates and allowing them to recover for 5-14 days (A-D). Assays were stopped when untreated cells became confluent. Quantification was done by Alamar blue staining (n=3). E-H. Limiting dilution assays after exposure to 100 μ M TMZ (closed square, dotted line for non-sphere cells; closed circle, continuous line for sphere cells) were performed and plotted against serum-free control (open squares, dotted line for non-sphere cells, open circle continuous line for sphere cells). Colonies or spheres were counted 4-20 days after

seeding (n=3, *P<0.05, ** P<0.01, ***P<0.001, 2 way ANOVA test) (NS, non-sphere cultures, SC sphere cultures).

Table 1. Characterization of non-sphere and sphere cell-derived tumors *in vivo*.^a

	SMA-497		SMA-540		SMA-560		GL-261	
	Non-sphere	Sphere	Non-sphere	Sphere	Non-sphere	Sphere	Non-sphere	Sphere
Early stage								
Median survival (mean±SEM)	14±0.13	>80	>80	>80	>80	15±0.15	35±0.53	73±2.2
Tumor incidence n/all (%)	23/23 (100)	1/7 (14)	3/7 (43)	0/7 (0)	2/4 (50)	13/14 (93)	4/4 (100)	4/4 (100)
Tumor diameter (mm)	2.8±0.1	0.17	0.7±0.04	n/a	0.85±0.11	3.9±0.20	3±0.49	3.7±0.35
Late stage								
Tumor incidence n/all (%)	n/a	5/14 (36)	9/16 (56)	1/16 (6)	6/13 (46)	None	8/16, 50	8/14, 57
Mean tumor diameter (mm)	n/a	1.6±0.05	3.9±0.32	3.7	2.9±0.5	n/a	4.0±0.21	3.1±0.31
Infiltration into contralateral hemisphere	yes	no	yes	no	no	Yes	yes	yes
Multifocal tumors (multifocal / total tumors, (%))	6/20, 30	0	3/8, 37.5	0	no	4/18, 22.2	4/18, 22.2	5/18, 27.8
Ki-67 mean ± SEM (%)	27.7±5.8	23.2±0.76 P=0.43	62.1±1.68	42.1±4.75	55.6±1.67	68.9±5.4* P=0.019	30.6±3.71	50.2±3.56 * P=0.032
Ki67 distribution within tumor	more at edge	n/a tumor too small	uniform	uniform	uniform	Uniform	uniform	uniform
CD31 count (mean±SEM)	42.8±2.97	44.4±2.62 P=0.70	41.5±1.63	39±0.49	86±2.59	97.3±4.32* P=0.011	47±2.05	44.6±2.91 P=0.20
CD3 count(mean±SEM)	451±69.7	155±40.1*** P=0.00036	88.4±36.6	67.5±36.3	122±46.3	113±41.1 P=0.07	178±23.9	359±51.3** P=0.03
CD11b count (mean±SEM)	1816±159	1015±142** P=0.0014	1927±180	1436±76	1386±133	1048±56 P=0.14	783±68	1334±139* P=0.032

^aSMA-497 non-sphere and SMA-560 sphere groups reached the terminal stage at day 14 (early stage) after implantation. To assess tumor morphology at progression, two to three mice per group were sacrificed in the other SMA groups. Results from 2 pooled experiments are included. Two mice per group were also sacrificed in the GL-261 studies at the occurrence of first symptoms on day 29. The remainder of the data included tumors that developed after day 14 up to and including the termination of the experimental day 80 after implantation (late stage). Quantitative data for Ki-67 stained cells is represented as the mean percentage of positive cells of the total along with the standard error of the mean (SEM) values. Quantification of blood vessel density (CD31), T cells (CD3) and microglial cells/macrophages (CD11b) is expressed as the mean and SEM of positively labeled cells (*P<0.05, **P<0.01, ***P<0.001, paired t-test with unequal variance due to heterogeneity within tumor areas). For all immune-labeled stainings, the cells were counted from 4-6 different areas (depending on tumor size) within the tumor from 3 different tumors (with the exception of the SMA-540 subline) each at the terminal stage in an area of 0.35 mm². All counts from all animals were derived to obtain mean and SEM. SMA-540 sphere cells were least tumorigenic. Because of the occurrence of only one incidence of tumor, statistical comparison between the non-sphere and sphere groups could not be made for all stainings done.